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(54) Title: METHOD FOR SUPPRESSION OF HIV REPLICATION BY ASCORBATE FOR CHRONIC AND ACUTE HIV INFECTION

(57) Abstract

A method is provided for treating symptomatic or nonsymptomatic conditions associated with HIV infection by inhibition of HIV replication, by administering to an infected subject a therapeutically-effective amount of ascorbate, ascorbic acid, their metabolic products, derivatives or mixtures thereof.

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METHOD FOR SUPPRESSION OF HIV REPLICATION BY ASCORBATE FOR CHRONIC AND ACUTE HIV INFECTION

The present invention is directed to a method for treating conditions associated with HIV infection by administering to an infected subject a therapeutic amount of ascorbate, or ascorbate in combination with other drugs efficacious for treatment of HIV-infection.

BACKGROUND

Previous studies demonstrated the antiviral activity of ascorbate against a broad spectrum of RNA and DNA viruses *in vitro* (Murata, et al., Agr. Biol. Chem., 36, 1065; 2597 (1972); Schwerdt, et al., Proc. Soc. Exp. Biol. Med., 148, 1237 (1975); Bissell, et al., P.N.A.S. USA, 77, 2711 (1980)) and *in vivo* (Klenner, J. Appl. Nutr., 23, 61 (1971); Cathcart, Biologisk Medicin, 3, 6 (1983)). Ascorbate was claimed to have inhibited the activation of a latent human retrovirus (HTLV-1) induced by 5-iodo-2'-deoxyuridine and N-methyl-N'-nitro-N-nitrosoguanidine (Blakeslee, et al., Cancer Res., 45, 3471 (1985)). However, it was not established whether ascorbate exerted a virus-specific effect or interacted directly with the activating substances. In addition, the ffects of ascorbate on acute inf ction by human retrovirus s or constitutive virus production associated with chronic infection hav not heretofor been determined. Oral and intravenous administration of ascorbate is said to have

produced clinical improvement in patients afflicted with influenza, hepatitis, and herpes virus infections, including infectious mononucleosis (Klenner, supra, and Cathcart, supra). In one study, some AIDS patients who voluntarily ingested high doses of ascorbic acid manifested clinical improvement (Cathcart, Medical Hypotheses, 14, 423 (1984)). The author attributed the effect to scavenging by ascorbate of free radicals produced by the disease and its associated infections.

Inhibitors of reverse transcriptase (RT) activity have been the focus of intensive investigation for the development of antiretroviral agents. Among these, 3'-azido-3'-deoxythymidine (AZT), the first drug approved for AIDS treatment, blocks de novo HIV infection but has recently been shown not to inhibit virus production in cells containing integrated HIV genomes. See Poli, et al., Science 244, 575 (1989). In the same study, interferon-α inhibited the budding and release of HIV from chronically infected cells stimulated with cytokines (TNF and PMA), but did not suppress constitutive virus production in unstimulated cells.

It is therefore an object of the present invention to provide a method for treatment of conditions associated with chronic and acute HIV infection by inhibition of HIV-replication, comprising the step of administering to a subject noncytotoxic ascorbate concentrations which are sufficient to inhibit virus replication.

It is yet a further object of the present invention to provide a method for treating both sympt matic conditions of HIV inf ction, such as AIDS, and nonsymptomatic conditions, such as ARC.

These and other objects will be apparent from the following description and appended claims and from the practice of the invention.

SUMMARY OF THE INVENTION

The present invention provides a method for combatting HIV infection by inhibition of HIV replication in a subject comprising the step of administering to the subject a therapeutically-effective amount of a compound selected from the group consisting of pharmaceutically-acceptable ascorbate salts; ascorbic acid; metabolic products of ascorbic acid or ascorbate salts; derivatives of ascorbic acid, ascorbate salts or the metabolic products thereof; and mixtures of two or more of any of the foregoing compounds.

DESCRIPTION OF THE FIGURES

Fig. 1 is a graph analysis of cytotoxicity of ascorbate for HTLV-III_B-infected H9 T-lymphocytic cells, as determined by trypan blue dye exclusion. Each point is the mean of four cell counts.

Fig. 2 shows the effect of ascorbate on reverse transcriptase (RT) activity in supernatant harvested from H9/HTLV-III_B cultures. In control samples, the RT values on day 2 and day 4 were, respectively, 55 x 10^4 and 267 x 10^4 cpm/10^6 cells; average background value in blanks (i.e., reactions without enzyme) was 1530 cpm per ml culture supernatant. In each experiment, the mean of three samples was determined and c mpared as a percentage of control (taken as 100%).

Fig. 3 shows th eff ct f ascorbate on HIV p24 antigen levels in supernatant harvested from H9/HTLV-III_B cultures. Extracellular p24 was assayed by Abbott HIV

antigen enzyme immunoassay. In control samples, the p24 levels on days 2 and 4 were, respectively, 244 and 45 nanograms/10^6 cells. The p24 values of ascorbate-treated cultures are compared as a percentage of control.

Fig. 4 is a graph of metabolic activity in H9 cells, as determined by MTT assay, in the presence and absence of ascorbate. Each point is the mean of four OD_{570} readings. Data are plotted as percentage of control.

Fig. 5 shows the protein synthesis rates in H9 cells in the presence and absence of ascorbate. Each point is the mean of ^{35}S-labeled amino acid incorporation per 10^6 cells.

Fig. 6 shows the dose-dependent decrease in HIV-induced syncytium formation with ascorbate. Syncytia were counted in CD_4^+ VB cells using a light microscope. Each point represents the mean of at least four samples and is compared as a percentage of the control infected cultures from the same experiment.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The active ingredient according to the present invention may be any pharmaceutically-acceptable ascorbate salt including, but not limited to, calcium, magnesium, potassium, or sodium salt. An active ingredient may also be ascorbic acid. Pharmaceutically-acceptable derivatives of ascorbic acid or ascorbate are also contemplated such as benzoylated ascorbate and other acylated ascorbates, palmitates or stearates. Metabolic products of ascorbic acid r ascorbates are also within the scope of the present invention, which m tabolic products include dehydroascorbate, dehydroascorbic acid, gulonolactone or gulonic acid and furan-type c mpounds that form adducts with amino and hydroxyl groups f

proteins. See Nakanishi, et al., Eur. J. Biochem., 152, 337 (1985); Garland, et al., Arch. Biochem. Biophys., 251, 771 (1986); and Ortwerth, et al., Exp. Eye Res., 47, 155 (1988).

Mixtures of ascorbate salts, ascorbic acid, metabolic products of ascorbic acid or ascorbate salts, derivatives of ascorbic acid or ascorbate salts or of the metabolic products are also contemplated to be within the scope of the present invention.

The above-described compounds will be administered in a therapeutically-effective amount to the HIV-infected subject. As described below, the effective amount of ascorbate inhibiting replication of HIV in vitro is greater than about 50 micrograms ascorbate/ml of cell growth medium and the cytotoxic amount is greater than about 400 μg/ml. The preferred methods for in vivo use of ascorbate in accordance with the present invention includes oral administration of preferably about 20 to 60 grams/day of ascorbate or other active compound within the scope of the present invention. It will be realized that this dosage level is approximate and may be exceeded since there is a high bowel tolerance for ascorbate.

Another preferred method of administration is by intravenous administration by drips or direct infusions. The useful dosage for intravenous injection is about 20 to 180 grams/day.

Alternatively, one or a mixture of compounds according to the present invention may b utilized, particularly in less than a therapeutically- ffective amount, when used in combination with other drugs used for treatment of HIV infection, such as AZT.

The method of the present invention is intended to be used for treatment of any condition associated with HIV infection whether that condition be symptomatic or nonsymptomatic of the infection. The primary symptomatic condition of HIV infection is AIDS whereas the primary nonsymptomatic condition of HIV infection is ARC.

It is surprising that ascorbate, ascorbic acid, their derivatives or metabolic products are useful for treatment of conditions associated with HIV infection. To validate the activity and mode of action of the compounds utilizing the method according to the present invention, the following tests were conducted on acutely and chronically HIV-infected T-lymphocytic cell lines grown continuously in the presence of nontoxic concentrations of ascorbate. Tests were conducted on the action of ascorbate on cell-free virus particles in vitro. The following methodology and experiments are presented for purpose of validation and illustration of the invention, but are not intended to limit the invention in any way.

MATERIALS AND METHODS

Cells and Cell Viability. H9 and H9/HTLV-III$_B$ cells (Popovic, et al., Science, 224, 497 (1984)) were originally obtained from Dr. Howard Streicher (National Cancer Institute, National Institute of Health). In some experiments, batches of the same cell lines provided by Dr. Michael McGrath, University of California at San Francisco, were also utilized, with identical results. Cells were grown in RPMI-1640 medium supplemented with 10% f tal calf serum, 2mM L-glutamine, 1mM pyruvate and 50 μg of gentamycin/ml. The CD4-positive VB cell line (Lifson, et al., Science, 232, 1123 (1986)) was propagat d in RPMI-1640 complete growth medium. Cell

viability was determined by using the trypan blue exclusion method.

Ascorbate. Stock solution of L-ascorbate was made by dissolving L-ascorbic acid (tissue culture grade from Sigma Chemicals) in RPMI-1640 medium, and was stored at -20°C.

Experimental Protocol. Fresh working solutions (10x strength) of ascorbate were prepared daily by diluting the stock in complete growth medium. For cytotoxicity assay, $3x10^5$ cells were suspended in 0.9 ml of growth medium and seeded in 24-well microtiter plates. Fresh solutions of ascorbate (0.1 ml of 10x strength) were added daily to obtain final concentrations of 10, 25, 50, 75, 100, 150, 200, 300, and 400 µg/ml. The controls received 0.1ml of growth medium. Plates were incubated at 37°C in 5% CO_2/95% air humidified atmosphere for various time intervals. At periodic intervals, 0.5 ml aliquots of cell suspension were collected, mixed with 50 µl trypan blue, and tested for viability.

For quantitation of viral and cellular parameters, cell suspensions (in triplicate) were collected, pooled, and centrifuged at 2000 rpm for 10 min. at 4°C. Supernatant was used for assays of extracellular RT activity and p24 antigen. Cell pellets were used for the determination of cellular metabolic activity and protein synthesis rates.

Assay of RT. Virus particles in supernatant were pelleted by centrifugation in a refrigerated microfuge (13,500 rpm, 2 hrs), then resusp nded in 1/50th of original volume of TNE buffer. Aliquots (10 µl) were assayed for RT activity as d scrib d by Hoffman, et al., using fresh batches of [methyl-^{3}H]-dTTP (5A ~80 Ci/mmol, NEN/Du Pont res arch products). RT activity was

expressed as the amount of [^{3}H]-dTMP incorporated (cpm/10^6 cells).

Assay of p24. Levels of p24 antigen in supernatant were assayed using the Abbott HIV antigen enzyme immunoassay (Goudsmit, Lancet ii, 177 (1986); Abbott Laboratories, North Chicago, IL). The p24 value was expressed as nanograms/10^6 cells for antigen released from infected cells or nanogram/ml for antigen contained in cell-free virus preparation.

Assay of Protein Synthesis. For radiolabeling, H9 cells (3 x 10^5 cells per well in microtiter plates) were grown in the presence of 0, 75, 100 and 150 μg/ml ascorbate as described earlier. On days 1, 2 and 4, cells were harvested, washed and resuspended in methionine- and cysteine-free medium and then incubated at 37°C for 30 min in 0.5 ml of the same medium supplemented with 50 μCi of ^{35}S-Translabel (5A 1013 Ci/Mm, ICN Radiochemicals). Labeled cells were pelleted, washed in phosphate-buffered saline, resuspended in lysis buffer containing 1% NP40, and stored at -70°C. Lysate was thawed and incubated at 100°C for 3-5 min to uncharge transfer RNA. Proteins were precipitated with trichloroacetic acid (TCA) in the presence of bovine serum albumin (0.2 mg per ml), transferred to nitrocellulose filters (0.45 μm), dried, suspended in βeta-blend (ICN Radiochemicals), and counted in a scintillation counter. Protein synthesis was determined on duplicate samples of cells independently grown in the presence of ^{35}S-labeled amino acids.

Metabolic Activity Assayed by MTT D t rmination. For metabolic activity assay, 3 x 10^5 cells were seeded in each w ll of 24-w ll microtiter plates and grown in the presence of 0, 75, 100, and 150 μg/ml ascorbate. On days 1, 2 and 4, cells wer pelleted, resuspended in 1.0 ml growth medium supplemented with 10% (v/v) MTT (3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, from Sigma Chemicals), incubated for 4 hrs, and treated with acidified isopropanol, and the absorbance at 570 nm was measured as described by Mossman, J. Immunol. Methods, 65, 55 (1983).

Inhibition Assay for the Cytopathic Effect of HTLV-III$_B$. Infectious HIV stock was obtained from supernatant fluid of H9/HTLV-III$_B$ cells cocultivated with VB cells at a 1:7.5 ratio for 3½ days. To quantitate syncytium formation, 2.5 x 10^5 VB cells in 0.4ml growth medium were mixed with 0.5ml HIV stock and seeded in 24-well microtiter plates. Then 0.1ml of either growth medium or 10x strength fresh L-ascorbate solution was added daily and the cells were incubated. On specific days after infection, total number of giant cell syncytia in each well were counted under the microscope using x100 magnification. A giant cell was defined as a cell >4 diameters larger than a single uninfected cell.

Cytotoxicity of Ascorbate

Before determining the effect on HIV production, the cytotoxicity of ascorbate on H9/HTLV-III$_B$ cells was evaluated, which are T-lymphocytic H9 cells infected with the AIDS virus (Popovic, supra). Ascorbate is unstable in solution as in conventional culture conditions, with a short half life, so an experimental protocol was adopted in which cell cultures were given daily additions of fresh solutions of ascorbic acid prepared in buffered growth medium (pH 7.3 ± 0.1). Cells were grown in the continuous presence of varying ascorbate concentrations (0 to 400 µg/ml) for a period of four days. Viability of control and ascorbate-tr ated cultures was determined using the trypan blue exclusion test. No toxicity was obs rved when cultures wer gr wn in the pr sence of 5 to 150 µg/ml ascorbate (Fig. 1). A slight inhibition of c ll growth (73-75% survival) was seen on the fourth day

of incubation in medium containing ascorbate at 200-300 μg/ml. Cytotoxicity became prominent (≥50% cell death) on the fourth day at ascorbate concentration of 400 μg/ml and higher. A slight increase in cell number was noted at concentrations ranging from 10 to 400 μg/ml on the first two days and at 5 to 75 μg/ml ascorbate on day 4. The remaining experiments evaluating ascorbate effects on HIV production were carried out at noncytotoxic concentrations of the compound.

Effects of Ascorbate on HIV Released from Chronically Infected Cells

a. Extracellular RT Activity in Supernatant. Reverse transcriptase (RT) activity was assayed in cell-free supernatant (Hoffman, et al., Virology, 147, 326 (1985)) harvested from cultures grown in non-toxic ascorbate concentrations (0 to 150 μg/ml). Fig. 2 shows the average of RT values of ascorbate-treated cultures and controls from 3 independent experiments. In the controls, RT titer manifested a peak of virus production on day 4. In contrast, ascorbate-treated cultures showed a striking inhibition of RT production. The first noticeable drop (64% inhibition) in RT titer occurred on day 2 at 50 μg/ml ascorbate, followed by a progressive decline in a dose-responsive manner. Further decreases in RT level were seen with increase in both ascorbate concentration and time of exposure. On day 4, over 99% inhibition in RT titer was seen at 150 μg/ml ascorbate. A noticeable increase in RT titer consistent with stimulation of cell growth was noted at low concentrations of ascorbate (from 5 to 25 μg/ml) on day 2. However, increase in virus production was transient, as these effects did not persist on day 4 of incubation.

b. p24 Levels in Supernatant. Another parameter of HIV production is the expression of p24 core antigen. Average values computed from three independent

experiments are presented in Fig. 3. Control cultures showed a rise in p24 antigen levels at day 2, reaching maximum levels on day 4. In contrast, p24 antigen expression was blocked in ascorbate-treated cultures. Concentrations of ascorbate required to inhibit p24 synthesis were higher than those effective in inhibiting RT production. Thus, the first significant reduction in p24 levels was seen at 150 μg/ml ascorbate on day 2. Higher declines in p24 values were observed with increase in time of exposure to ascorbate. On the fourth day, p24 levels in cultures treated with 150 μg/ml ascorbate were reduced to 13% of the control.

Effect of Ascorbate on Cell Metabolism

The following work addressed the question of whether ascorbate-induced suppression of RT and p24 production in H9/HTLV-III_B cells was a virus-specific effect or an indirect effect due to inhibition of cellular metabolism or protein synthesis. The metabolic activity of uninfected H9 cells in the presence and absence of ascorbate was determined by using a quantitative colorimetric assay that utilizes the tetrazolium salt MTT (Mossman, J. Immunol. Methods, 65, 55 (1983)). This salt measures the activity of various dehydrogenases in viable cells (Montagnier, et al., AIDS Res. Hum. Retroviruses, 4 (6), 441 (1988)). H9 cells grown in the presence of different concentrations of ascorbate (0 to 150 μg/ml) showed an increase in cellular metabolic activity on day 1 (Fig. 4). This correlated with stimulation of cell proliferation by ascorbate. On days 2 and 4, no significant change in metabolic activity was not d between control cultures and those exposed to ascorbate at conc ntrations of 75, 100, and 150 μg/ml.

Effect of Ascorbat on Cellular Prot in Synthesis

The effect of ascorbate on cellular protein synthesis was determined by growing uninfected H9 cells for 4 days

at concentrations of 0, 75, 100, and 150 μg/ml (Somasundaran, et al., Science, 242, 1554 (1988)). On day 1, ascorbate was observed to stimulate protein synthesis, consistent with stimulation of metabolic activity and cell growth. On days 2 and 4, there was a less than 2-fold difference in the apparent rates of cellular protein synthesis in both ascorbate-treated and control cultures (Fig. 5). Thus the suppressive effects on HIV production could not be ascribed to a general inhibition of cellular metabolism or protein synthesis.

Effect of Ascorbate on Virus Replication in Freshly Infected Cells

To extend these findings to freshly infected cells, we investigated the effects of ascorbate on acute HIV infection of susceptible $CD4^+$ T-lymphocytes. Viral infectivity and cytopathic effect in these cells have been correlated with formation of giant-cell syncytia mediated by interaction of HIV envelope glycoprotein with $CD4^+$ cell surface receptor. In controls, multinucleated syncytia became visible by day 4, reaching high levels on day 6. The continuous presence of ascorbate in the growth medium of infected cells caused a dose- and time-dependent decrease in syncytium formation. On day 4, approximately 93.3% inhibition in syncytia number was seen at 100 μg/ml ascorbate (Fig. 6). At this concentration, ascorbate did not inhibit the growth of uninfected VB cells (99% survival by trypan blue dye exclusion), indicating that the inhibition of virus replication was not due to cytotoxic effect of the compound.

Direct Inactivation of Virus Particles in Supernatant

The following work addressed the anti-HIV mechanism to determine whether decrease in RT titer and syncytium formation were due to direct inactivation of virus particles by ascorbate in vitro. Cell-free supernatant containing infectious virus was prepared and incubated in the presence and absence of ascorbate at 37°C for 8 and 18 hrs. Samples were tested for RT activity and syncytium formation was measured in VB cells. After incubation at 37°C for 18 hrs, there was no detectable difference in RT activity between ascorbate-treated virus preparations and controls (Table 1). Syncytium-forming titer of infectious virus of ascorbate-treated and untreated preparations after incubation at 37°C for one day was also approximately equal (2.34-2.70 x 10^3 $TCID_{50}$/ml). When chronically infected cells were exposed to 150 μg/ml ascorbate for 18 hrs at 37°C, the RT titer in culture supernatant was reduced to 11.2% of the control (Table 1). These results indicate that decrease in extracellular RT titer, first seen after overnight treatment of chronically infected cells by ascorbate, was not due to direct inactivation of cell-free virus.

To study RT stability further in the presence of ascorbate upon prolonged incubation (37°C for several days), the following experiment was carried out. Since thermal inactivation of cell-free virus occurs upon extensive incubation at 37°C, uninfected cells were used to protect virus from heat inactivation. These conditions resemble those present in experiments utilizing HIV-infected cell lines that were grown in the continuous presence of ascorbat for several days. Accordingly, HIV supernatant was mixed with uninfected VB cells and incubat d with asc rbat for 4 days, with daily addition of fr sh compound. Supernatants were harvested and assayed for RT activity. Aft r 4 days in

the presence of 100 and 150 μg/ml ascorbate, RT activity was reduced respectively to 31.5% and 7.0% of control (Table 1). In parallel experiments, chronically infected cells were exposed to 100 and 150 μg/ml ascorbate for 4 days. The RT levels in supernatant were reduced to 4.0 and 0.6% of control (Table 1).

In another experiment, the stability of p24 antigen was compared in the presence and absence of ascorbate at 37°C for 4 days. After incubation of cell-free virus with 150 μg/ml ascorbate, the concentration of p24 protein in the ascorbate-treated preparation (283 ng/ml) was not significantly different from that of the control (263 ng/ml), indicating that the compound does not cause loss of p24 antigenicity in vitro. At the same ascorbate concentration, chronically infected cells exhibited ~8-fold reduction in p24 antigen production after 4 days at 37°C (Fig. 3).

Table 1. Analysis of RT stability and RT production in the presence of and absence of ascorbate.

Virus/cell source	Ascorbate concentration (μg/ml)	8 hrs		18 hrs		4 days	
		cpm x 10^{-4}/ 10^6 cells	% control	cpm x 10^{-4}/ 10^6 cells	% control	cpm x 10^{-4}/ 10^6 cells	% control
HIV Supernatant	0	6.68	100	6.06	100	ND	ND
	100	7.00	105	6.17	102	ND	ND
	150	7.40	111	6.81	112	ND	ND
HIV-VB Suspension	0	ND	ND	16.4	100	5.86	100
	100	ND	ND	12.9	78.4	1.85	31.5
	150	ND	ND	7.84	47.8	0.41	6.96
H9/HTLV-III$_B$ Supernatant	0	79.2	100	56.7	100	267	100
	100	81.9	103	12.7	22.4	10.6	3.96
	150	67.5	85.2	6.33	11.2	1.54	0.58

HIV virus supernatant was prepared from H9/HTLV-III$_B$ cells. Virus supernatant alone or a suspension of supernatant and uninfected VB cells (3 x 10^5 cells per ml) were exposed to 0, 100 and 150 μg/ml asc rbate and incubated at 37°C with daily addition of fresh compound. In a parallel xperiment, chronically-infected H9/HTLV-III$_B$ were grown under similar conditions. At different time p riods, supernatants were collected and assayed for RT activity as described in Materials and Meth ds. ND = not done.

WHAT IS CLAIMED IS:

1. A method for combatting HIV-infection in a subject having a condition which is nonsymptomatic of said infection, by inhibition of HIV replication comprising the step of administering to said subject a therapeutically-effective amount of a compound selected from the group consisting of pharmaceutically-acceptable ascorbate salts; ascorbic acid; metabolic products of ascorbic acid or said salts; derivatives of ascorbic acid, said salts or said metabolic products; and mixtures thereof.

2. A method according to Claim 2 wherein said condition is ARC.

3. A method for controlling HIV-infection in a subject having a condition which is symptomatic of said infection, by inhibition of HIV replication, comprising the step of administering to said subject a therapeutically effective amount of a compound selected from the group consisting of metabolic products of ascorbic acid or pharmaceutically acceptable ascorbate salts, derivatives of ascorbic acid or pharmaceutically acceptable ascorbate salts, and mixtures thereof.

4. A method according to Claim 3 wherein said condition is AIDS.

5. A method according to Claim 1 or 3 wherein said administration is oral.

6. A method according to Claim 1 or 3 wherein said administration is intraven us.

7. A method according to Claim 1 or 3 wherein said administration is oral and intravenous.

8. A method according to Claim 5 wherein said therapeutically-effective amount is in the range of about 20 to 180 gm/day.

9. A method according to Claim 6 wherein said therapeutically-effective amount is in the range of about 20 to 180 gm/day of ascorbate.

10. A method for treating a condition associated with HIV infection in a subject by inhibition of HIV replication comprising the step of administering to said subject a therapeutically-effective amount of a combination of:

(a) a compound selected from the group consisting of pharmaceutically-acceptable ascorbate salts; ascorbic acid; metabolic products of ascorbic acid or said salts; derivatives of ascorbic acid, said salts or said metabolic products; and mixtures thereof; and

(b) at least one other drug efficacious for treatment of symptomatic or nonsymptomatic HIV-infection.

11. A method according to Claim 11 wherein said drug comprises AZT.

12. A method according to Claim 10 wherein said condition is nonsymptomatic of HIV-infection.

13. A method according to Claim 12 wherein said condition is ARC.

14. A method according to Claim 10 wherein said condition is symptomatic of HIV infection.

15. A method according to Claim 14 wherein said condition is AIDS.

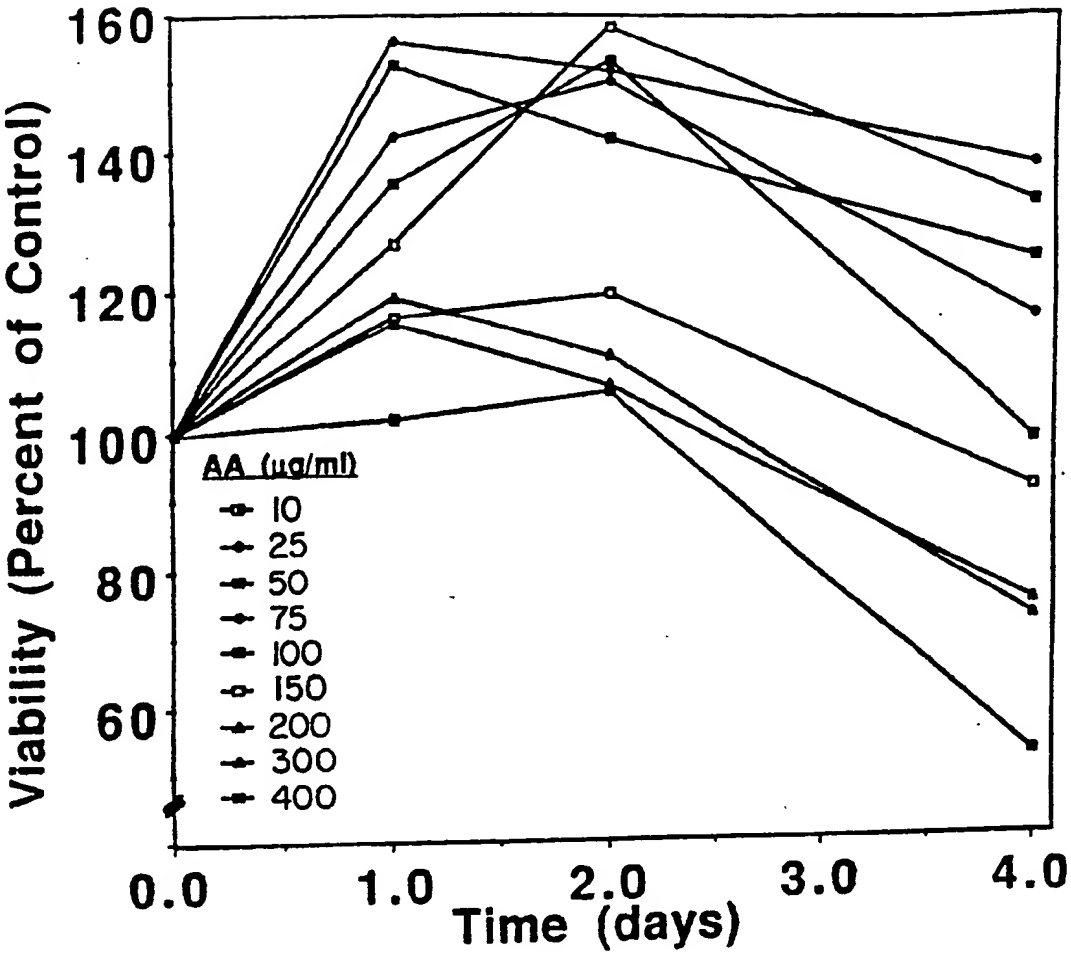
160
140
120
100
80
60
Viability (Percent of Control)
AA (µg/ml)
10
25
50
75
100
150
200
300
400
0.0
1.0
2.0
3.0
4.0
Time (days)

Fig. 1

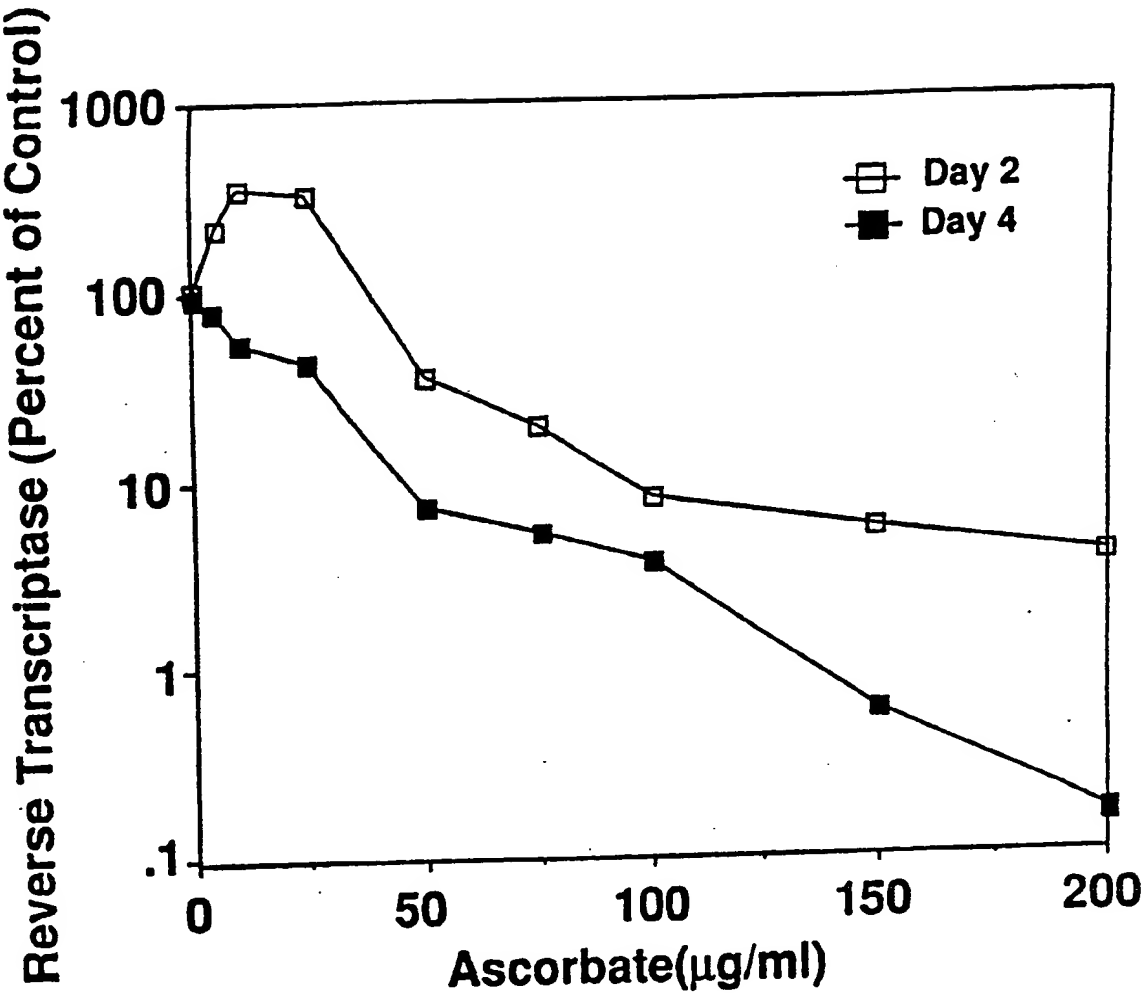
Reverse Transcriptase (Percent of Control)
1000
100
10
1
.1
0
50
100
150
200
Ascorbate(µg/ml)
Day 2
Day 4

Fig. 2

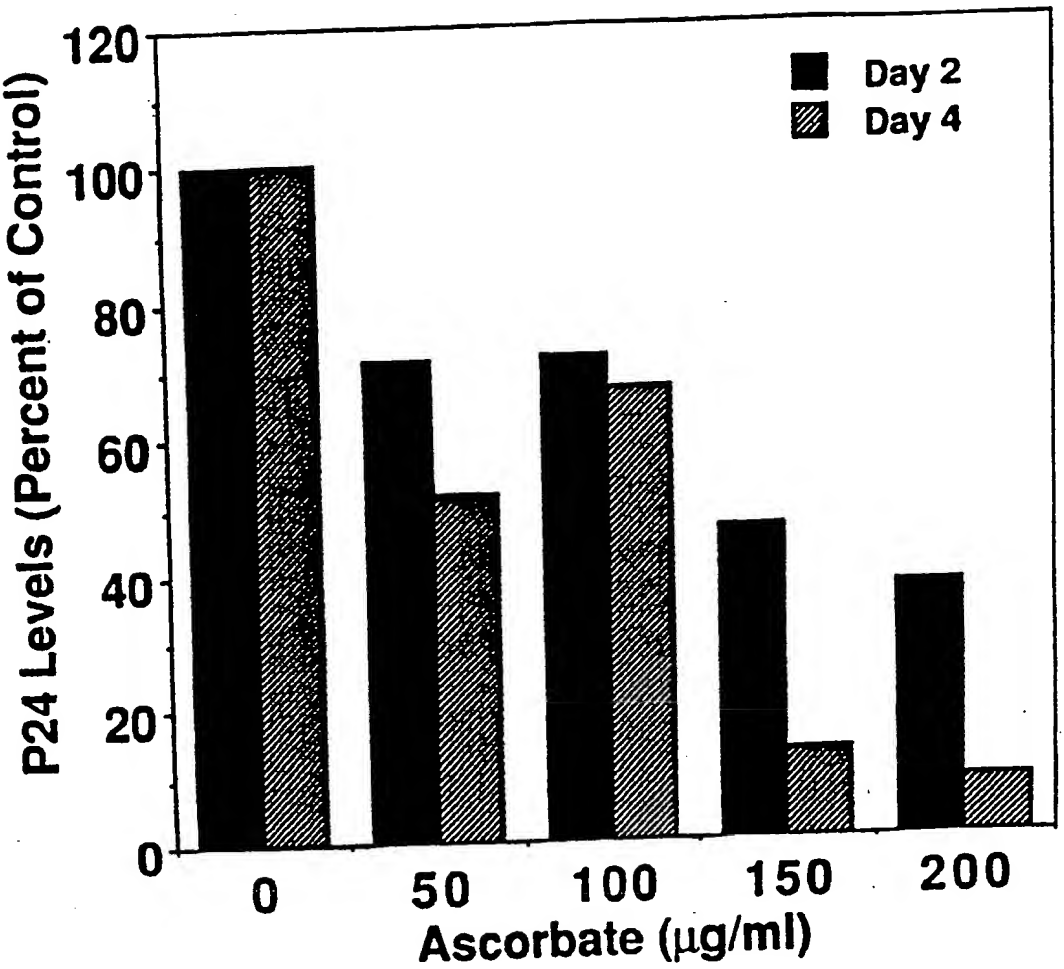
120
100
80
60
40
20
0
P24 Levels (Percent of Control)
Day 2
Day 4
0
50
100
150
200
Ascorbate (µg/ml)

Fig. 3

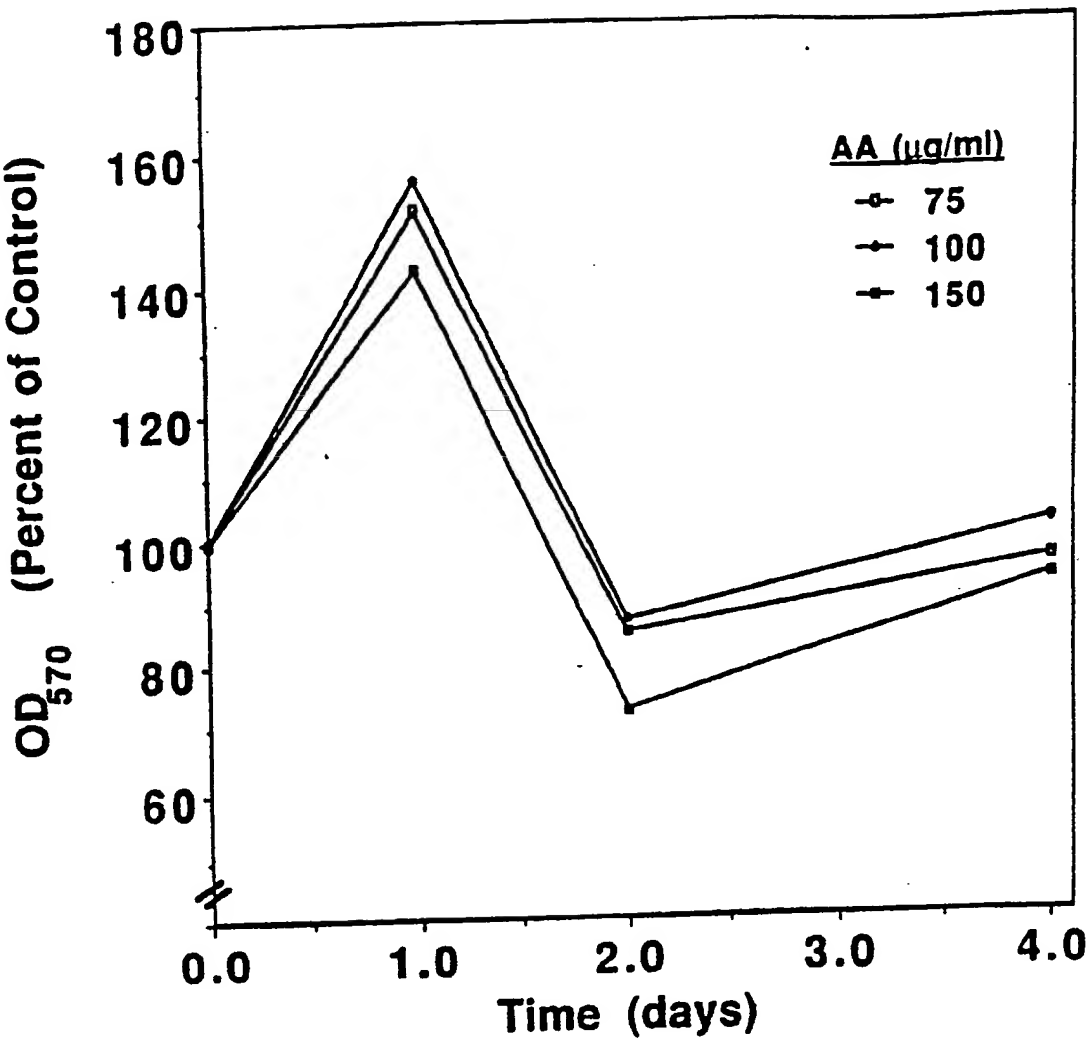
180
160
140
120
100
80
60
OD570 (Percent of Control)
AA (µg/ml)
75
100
150
0.0
1.0
2.0
3.0
4.0
Time (days)

Fig. 4

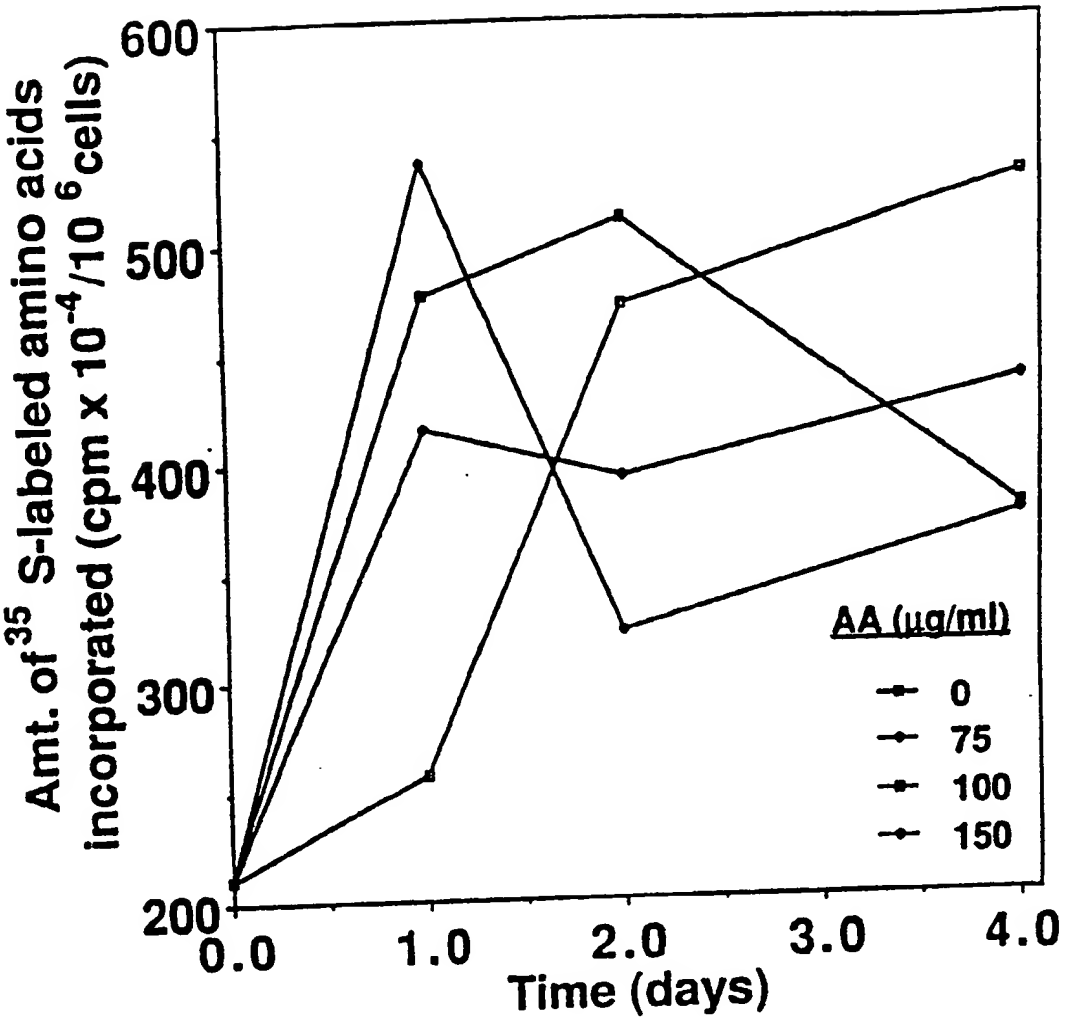
Amt. of 35 S-labeled amino acids incorporated (cpm x 10-4/10 6cells)
600
500
400
300
200
0.0
1.0
2.0
3.0
4.0
Time (days)
AA (µg/ml)
0
75
100
150

Fig. 5

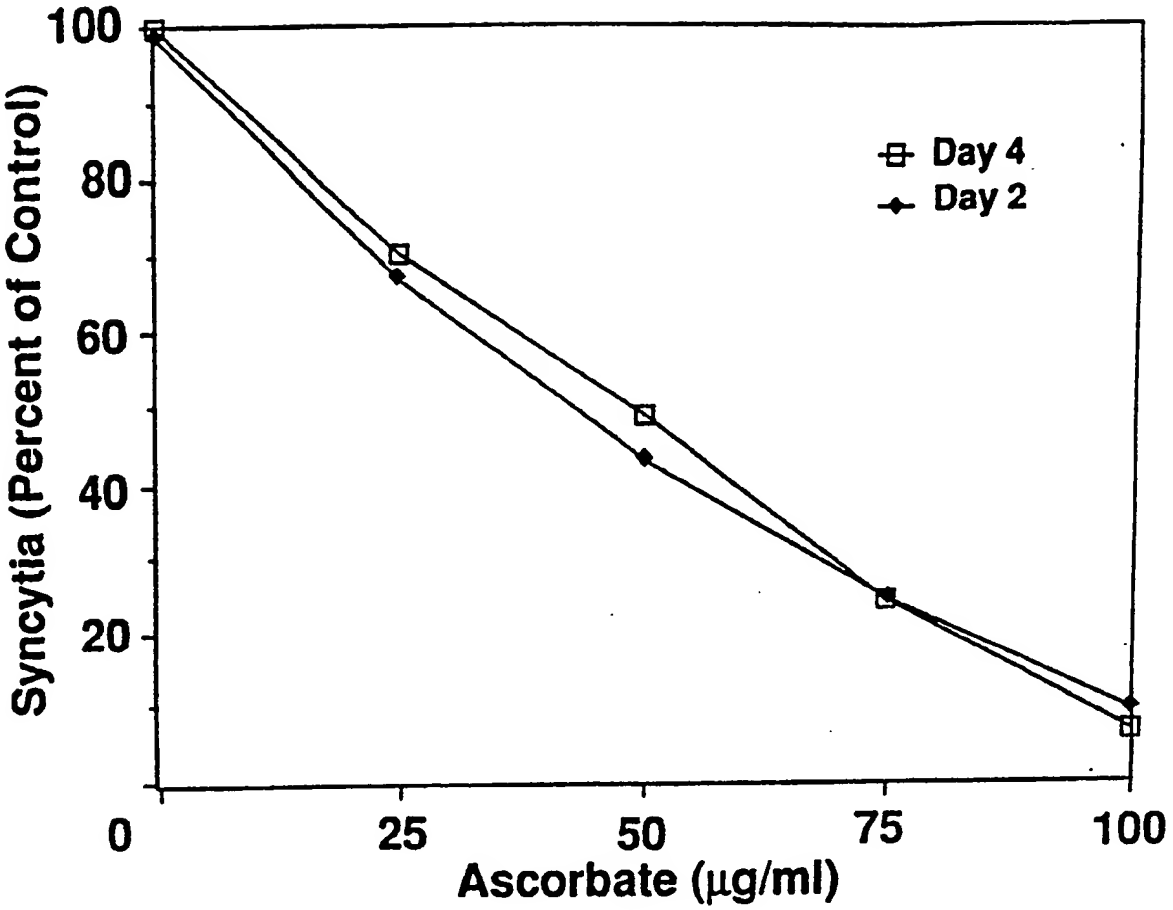
100
80
60
40
20
0
25
50
75
100
Syncytia (Percent of Control)
Ascorbate (µg/ml)
Day 4
Day 2

Fig. 6

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05895

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A01N 43/08
US CL : 514/474

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	514/474

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CA: 111 (19): 167396 f- Treatment acquired immunodeficiency especially AIDS, with 17-(cyclopropylmethyl) -4,5-epoxy-3,14-dihydroxymorphenon -6-one and pharmaceutical compositions containing it-. See entire document.	1-15
A	CA: 110(6): 44968 r -Vaccines containing mycobacterium phlei FU and diisopropyl-ammonium-dichlorate and ascorbic acid for the treatment of HIV infections See entire document.	1-15
A	Cathcart, Biologisk Medicine, 3:6 (1983), See entire statement.	1-15
X,Y	Cathcart, R.F. Medical Hypothesis, 14:423-433 (1984) See entire article.	1-15
A	Nakanishi, Y. et al, European Journal of Biochemistry 152:337-342 (1985) - see entire document.	1-15

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 OCTOBER 1991	08 NOV 1991

International Searching Authority	Signature of Authorized Officer
ISA/US	INTERNATIONAL DIVISION THEODORE J. CRIARES

Form PCT/ISA/210 (second sheet) (Rev.11-87)

DC 11-01-91

FURTHER INFORMATION CONTINUED FROM THE SEC NO SHEET

A	Garland, D., et al. Archives of Biochemistry and Biophysics, 251:7710776 (1986) See entire article	1-15
A	Ortwerth, B.J. et al, Exp. Eye Res., 47: 155-168 (1988) See entire article.	1-15

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons.

1. ☐ Claim numbers because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [4]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.